Cell entry mechanisms of dengue virus
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Immature Dengue Virus: A Veiled Pathogen?

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Chapter 5

Abstract

Dengue virus is an emerging pathogen which may cause severe illness upon secondary infection, presumably due to antibody-dependent enhancement of disease. It was recently described that patients who develop severe disease have elevated levels of antibodies against the prM protein. Also, cells infected with dengue virus release substantial amounts of prM-containing immature virus. These observations suggest that immature particles play a role in dengue pathogenesis. However, functional studies have demonstrated that immature particles lack the ability to infect cells. Here, we show that immature dengue virus becomes highly infectious in the presence of anti-prM antibody. The antibody facilitates entry of immature dengue virus into cells carrying Fc receptors. Furthermore, furin activity in target cells is critical for triggering infectivity of immature virus. These data indicate that in the presence of anti-prM antibody immature dengue virus has the potential to become highly infectious and thus may contribute to the development of severe disease.
Dengue virus (DENV) represents a major emerging arthropod-borne pathogen. There are four distinct serotypes of DENV which according to WHO estimates infect about 50-100 million individuals annually, mostly in the (sub)tropical regions of the world. While most DENV infections are asymptomatic or result in self-limited dengue fever (DF), an increasing number of patients present more severe, potentially fatal clinical manifestations, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). It is well established that a major risk factor for the development of DHF/DSS is secondary infection with a heterotypic virus serotype (1-3). Also primary infection in infants born to dengue-immune mothers may lead to severe disease (4, 5). These observations have led to the now widely accepted hypothesis of antibody-dependent enhancement (ADE) of infection (6). Increased disease severity appears to correlate with high circulating virus titers (3, 7), suggesting that antibodies directly influence the infectious properties of the virus. The molecular mechanism by which antibodies enhance DENV infection however remains elusive.

DENV, as well as other major human pathogens like West Nile virus (WNV), yellow fever virus, tick-borne encephalitis (TBEV), and Japanese encephalitis virus, belong to the Flavivirus genus within the family Flaviviridae. The Flavivirus genus comprises small (~50 nm in diameter) spherical enveloped virions which contain a single-stranded positive-sense RNA genome packed together with multiple copies of the capsid protein (8). The viral envelope anchors two glycoproteins, the envelope protein E, which is organized in 90 homodimers on the viral surface, and the small membrane protein M (9). DENV enters its host cell via clathrin-mediated endocytosis and fuses from within acidic endosomes, through which the viral genome gains access to the cytoplasm (10). Following RNA replication and protein translation, immature virions, which contain heterodimers of the E protein and a precursor form of M (prM), are assembled within the ER. Subsequently, the particles mature by passing through the Golgi and trans-Golgi network (TGN) (11). In the acidic environment of the TGN, the virion undergoes a conformational change and the cellular endoprotease furin cleaves prM into M and a peptide (“pr”) that remains associated with the virion (12). Upon release, the pr peptide dissociates from the virion, resulting in the formation of mature progeny virions.

Humoral immunity of DENV-infected individuals is mainly directed against the E and prM glycoproteins. In vitro studies have revealed that E-specific antibodies enhance infection when the number of bound antibodies does not exceed the threshold required for virus neutralization (13). Antibodies
against the E protein have been observed to facilitate efficient cell entry of virions via the Fc receptor, leading to increased virus particle production. While the role of E-specific antibodies has been studied scrupulously, the occurrence and importance of antibodies against the prM protein remain elusive. The existence of prM-specific antibodies in DENV-infected human sera correlates with multiple studies showing that DENV-infected cells secrete high numbers (~30%) of immature prM-containing virions (14-19). These findings together with the recent observation that DHF/DSS patients have elevated levels of anti-prM antibody responses suggest that immature particles somehow contribute to disease pathogenesis (20). On the other hand, extensive functional analyses of immature virions have revealed that immature flaviviruses lack the ability to infect cells, as the presence of uncleaved prM in the virion blocks the E glycoprotein from undergoing the pH-induced conformational changes that are required for membrane fusion (19, 21-25). If indeed uncleaved prM renders DENV non-infectious, how might immature particles then contribute to disease pathogenesis and what could be the role of anti-prM antibodies in the enhancement of infection?

In an attempt to answer this question, we investigated the infectious properties of immature DENV-2 virions in Fc receptor-expressing K562 cells in the absence or presence of anti-prM antibody 70-21. This is an IgG2a antibody that has been isolated from DENV-infected mice and is mapped to amino acids 53-67 of prM (26). Antibodies recognizing this epitope are abundantly present in sera of DHF/DSS patients (20). Immature DENV particles were generated in furin-deficient LoVo cells. We previously determined that these particles are fully immature with an average content of 94% ± 9% prM (19). K562 cells were incubated with immature DENV particles in the absence and presence of increasing concentrations of the 70-21 antibody at a multiplicity of 100 genome-containing particles per cell (MOG 100). The number of genome-containing particles (GCP) was determined by quantitative PCR analysis of reverse-transcribed viral RNA (14). At 43 h post-infection (hpi), cells were fixed and prepared for flow cytometric analysis to determine the number of infected cells, measured on the basis of dengue E protein expression. In agreement with the literature (12, 19, 21, 24), we found that immature particles are non-infectious as the number of E-positive cells did not exceed the limit of detection (Fig. 1A). Remarkably however, substantial numbers of E-positive cells were observed upon infection with immature particles opsonized with the anti-prM antibody (Fig. 1A). Subsequent titration of the cell supernatants at 43 hpi revealed that opsonization of immature DENV with anti-prM antibody
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Figure 1. Immature DENV particles become infectious in the presence of anti-prM antibodies. K562 cells were infected with immature (prM) or wild-type DENV-2 (strain 16681) at MOI 100 in the presence or absence of anti-prM (70-21). (A) Representative readout of the percentage of infected cells. Prior to infection, immature DENV particles were incubated with 40 ng/ml 70-21 antibody for 1 h at room temperature. At 43 hpi, the cells were fixed, stained intracellularly with Alexa-647-coupled anti-E antibody 3H5.1, and subjected to flow-cytometric analysis. Mock-infected cells and an IgG isotype control (murine IgG2a) antibody were used as controls (B) Titration of the virus particles released at 43 hpi from infected K562 cells was performed by plaque assay on BHK-15 cells. Data are expressed as means of at least three independent experiments. The error bars represent standard deviations (SD); (n.d.) denotes “not detectable”.

dramatically enhanced (up to 30,000-fold) virus particle production (Fig. 1B). Indeed, prM-opsonized immature particles were nearly as infectious as wild-type DENV (Fig. 1B). Enhancement of immature DENV infectivity was seen at a broad antibody concentration range, even at conditions of high antibody excess.

To better understand the mechanism by which prM antibodies trigger infectivity of immature DENV, we analyzed the distinct steps in the cell entry...
pathway of the virus. First, the binding of immature virions to K562 cells was determined by quantitative PCR. In order to determine the number of bound GCP per cell, the amount of virus added per cell was increased 10-fold compared to the concentration used in the infectivity experiments. The results show that antibody-opsonized immature DENV binds approximately 30-fold more efficiently to cells than immature particles in the absence of antibody (Fig. 2A). Moreover, it appeared that immature particles opsonized with anti-prM bind almost as efficiently to cells as wild-type DENV. It is likely that binding of virus-antibody complexes is mediated by direct interaction of the antibody with the Fc receptor (FcR) expressed on the cell surface. Indeed, treatment of cells with an anti-CD32 antibody to block the FcγIIIR interaction, severely reduced virus particle production upon infection of K562 cells with opsonized immature virions, whereas it had no effect on infection with wild-type virus (Fig. 2B). Collectively, these data indicate that prM antibodies facilitate efficient interaction and cell entry of virus-immune complexes via the FcγIIIR, explaining at least partially the observed stimulation of infectivity of immature DENV virions.

However, efficient FcγIIIR-mediated cell entry does not clarify what the trigger is for immature virions to become infectious, since the presence of prM

Figure 2. Anti-prM antibody stimulates binding of immature DENV particles to cells through interaction with FcγIIIR. (A) Binding of immature and wild-type virions with and without prior opsonization to antibodies (40 ng/ml) to K562 cells. Virus-cell binding was measured after a 1 h incubation at 4°C by quantitative PCR analysis. (B) Effect of anti-CD32, which blocks interaction with FcγIIIR, on virus particle production. Virus particle production was determined as described in the legend to Figure 1B. Data are expressed as means and SD of three independent experiments. Two-tailed Student’s t-tests were performed for statistical analysis of the data.
has been shown to obstruct membrane fusion activity of the virus (12, 19, 21). One could speculate that anti-prM antibodies bound to immature virions induce a conformational change that would enable the E protein to trigger membrane fusion irrespective of the presence of prM. Another scenario may be that prM-containing virions mature upon cell entry since furin, although predominantly present in the TGN, also shuttles between early endosomes and the cell surface. To verify the potential involvement of furin during virus cell entry, we investigated the infectious properties of antibody-opsonized immature DENV in cells treated with furin inhibitor, decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (decRRVKR-CMK). In aqueous solution, decRRVKR-CMK has a half-life of 4-8 h (27), and therefore is not expected to interfere with the maturation process of newly assembled virions within the infected cell. The results show that inhibition of furin activity completely abrogated virus particle production in cells infected with antibody-opsonized immature virions, whereas infection of cells with wild-type virus remained unaffected under these conditions (Fig. 3). Collectively, these results demonstrate that furin activity in target cells plays a vital role in triggering infectivity of antibody-opsonized immature DENV after FcγII receptor-mediated cell entry of the virus.

**Figure 3. Immature DENV particles mature upon FcγIIIR-mediated cell entry.** K562 cells were infected with DENV or DENV-immune complexes in the presence or absence of furin inhibitor (25 μM). As a positive control for compound activity, wild-type DENV-infected cells were treated with an additional dose of furin at 24 hpi to impede virus maturation and consequently the production of infectious virions (++). Virus particle production was determined as described in the legend to Figure 1B. Data are expressed as means and SD for three independent experiments; (n.d.) denotes “not detectable.”
In this study, we demonstrate that immature DENV, which is generally considered to be non-infectious, becomes highly infectious upon opsonization with anti-prM antibodies. Our data indicate that anti-prM antibody facilitates efficient binding and entry of immature particles into Fc receptor-expressing cells. Furthermore, it is demonstrated that furin activity within the target cells is involved triggering viral infectivity presumably through cleavage of the prM protein.

Multiple studies have shown that immature particles are non-infectious, the presence of prM obstructing the low-pH-induced conformational changes in the viral E glycoprotein required for membrane fusion of the virus (12, 21, 23, 24, 28). In this report, we show that the lack of infectivity is primarily related to inefficient binding of immature virions to the cell surface. If binding is facilitated through anti-prM antibodies, immature DENV virus particles become highly infectious presumably due to efficient intracellular processing of prM to M by the endoprotease furin.

It is likely that DENV maturation occurs within acidic endosomes, since previous in vitro experiments have revealed that cleavage of immature particles by furin is dependent on the exposure of the virus to low pH (12). We propose that the acidic conditions of the endosome, similar to those in the acidic TGN during processing of newly assembled virions, triggers an initial conformational change in the virion such that furin is able to cleave prM to M and the "pr" peptide. Interestingly, a recent report study has shown that upon cleavage of prM a large fraction of pr peptide remains associated with the virion and that back-neutralization to pH 8.0 is required to release the pr peptide from the virion (12). The authors interpreted this as a mechanism preventing newly assembled cleaved virions from undergoing membrane fusion in the acidic TGN. However, this notion is difficult to reconcile with our present observations, since virions that have matured within acidic endosomes of target cells do not return to neutral-pH conditions before initiating infection. One may speculate that the pr peptide stabilizes the E protein to such an extent that it survives the mildly acidic lumen of the TGN (pH ~6.0), but is released at the more acidic pH of endosomes (pH 5.5-5.0) such that the E proteins have the capacity to rearrange to the fusion-active conformation. Another possibility is that upon cleavage of prM the pr peptide associates with the anti-prM antibody instead of the E protein.

The observed infectious potential of immature DENV virions in the presence of anti-prM antibodies may have important implications for our understanding of the processes involved in dengue pathogenesis. We postulate
that in the early stages of a primary infection, before the appearance of virus-specific antibodies, immature virions fail to penetrate host cells and therefore are of minor significance in disease development. On the other hand, during a secondary infection or primary infection of infants born to dengue-immune mothers, immature particles may become highly infectious due to the presence of anti-prM antibodies and hence may contribute to an increased dengue-infected cell mass and a high circulating virus titer, one of the preludes for the development of severe disease symptoms (3, 7, 29). Importantly, anti-prM antibodies may activate the infectious properties of a large population of virus particles, since we recently observed that a typical DENV-2 preparation of the prototype strain 16681 contains as much as 30% prM (19). The hypothesis that immature particles act as an veiled pathogen is strengthened by a recent report which shows that DHF/DSS patients have elevated antibody levels directed against the prM protein (20). Interestingly, a large fraction of these prM antibodies recognized the same epitope as the 70-21 antibody used in this study.

The observation that anti-prM antibody enhances rather than neutralizes the infectivity of immature particles even at conditions of high antibody excess may also have important repercussions for vaccine development. Generation of antibodies against the prM protein activates the infectious properties of immature DENV particles and therefore may have adverse effects on protection from infection. Future research is required to probe antibody responses against vaccine candidates and to determine which types of antibodies are required for protection.
Chapter 5

Materials and methods

Cells

*Aedes albopictus* C6/36 cells were maintained in minimal essential medium (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, 7.5 % sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 μg/ml), 200 mM glutamine and 100 μM nonessential amino acids at 28°C, 5 % CO₂. Baby Hamster Kidney-21 clone 15 cells (BHK-15) cells were cultured in DMEM (Life Technologies) containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), 10 mM HEPES, and 200 mM glutamine at 37°C, 5% CO₂. Human adenocarcinoma LoVo cells were cultured in Ham’s medium (Life Technologies) supplemented with 20% FBS at 37°C, 5% CO₂. Human erythroleukemic K562 cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C, 5% CO₂.

Virus growth

DENV-2 strain 16681, kindly provided by dr. Claire Huang (Center for Disease Control and Prevention, USA), was propagated on C6/36 cells as described before (19). Briefly, monolayers of C6/36 cells were infected at multiplicity of infection (MOI of 0.1). At 96 hpi, the medium was harvested, cleared from cellular debris by low-speed centrifugation, aliquoted, and stored at -80°C. Immature DENV particles were produced on LoVo cells as described previously (19). Briefly, LoVo cells were infected at MOI 10. Virus inoculum was removed after 1.5 h and fresh medium was added after washing the cells twice with PBS. At 72 hpi, the medium containing the virus particles was harvested, cleared from cellular debris by low-speed centrifugation, aliquoted, and stored at -80°C. Virus preparations were analyzed with respect to the infectious titer and the number of genome-containing particles, as described previously (14, 19).

Infectivity assays

Virus or virus-antibody complexes were added to 2 × 10⁵ K562 cells, at a multiplicity of 100 genome-containing particles (MOG) per cell. After 1.5 h incubation at 37°C, the inoculum was removed and fresh medium was added to the cells. At 43 hpi, the medium was harvested and virus production was analyzed by plaque assay on BHK-15 cells, as described previously (30). To measure the number of infected cells, cells were fixed at 43 hpi, stained with 3H5-conjugated Alexa647, and analyzed using a FACS Calibur cytometer. For virus-antibody complex formation, virus particles were incubated for 1
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h at 37°C with various dilutions of monoclonal prM antibody 70-21 (mAb 70-21) in K562 medium containing 2% FBS prior to the addition to cells. When indicated, K562 cells were pretreated with 25 μg/ml of anti-FcγRII antibody (MCA1075PE, Serotec, Oxford, United Kingdom) for 1 h at 37°C, after which access antibody was removed by extensive washing. In furin blockage experiments, 25 μM of decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (decRRVKR-CMK) (Calbiochem, Darmstadt, Germany) was added prior and during virus infection.

Binding assays
To determine the number of bound genome-containing particles per cell, virus or virus-antibody complexes were incubated with 2 × 10⁵ K526 cells at MOG 1000 for 1 h at 4°C. Subsequently, cells were washed three times with ice-cold PBS containing MgCl₂ and CaCl₂ (Life Technologies) to remove unbound virus-antibody complexes. Then, viral RNA was extracted from the cells by use of the QIAamp Viral RNA mini Kit (QIAGEN, Venlo, The Netherlands). Thereafter, cDNA was synthesized from the viral RNA with reverse transcription-PCR (RT-PCR), copies of which were quantified using quantitative PCR (14).
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References

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